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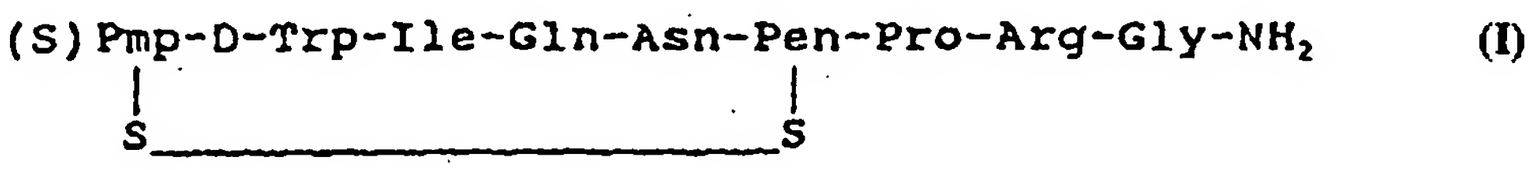
(54) Title: OXYTOCIN ANTAGONIST

(57) Abstract

The oxytocin antagonist represented by formula (I) wherein (S-Pmp IS β,β-(3-thiapentamethylene)-β-mercaptopropionic acid, D-Trp is the D form of tryptophan, and Ile, Gln, Asn, Pen (Pen = penicillamine), Pro, and Arg are the L forms of isoleucine, glutamine, asparagine, proline and arginine, respectively. This compound can be administered to pregnant women to arrest premature labor while avoiding unwanted side effects due to antagonism of the antidiuretic hormone, vasopressin.



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OXYTOCIN ANTAGONIST

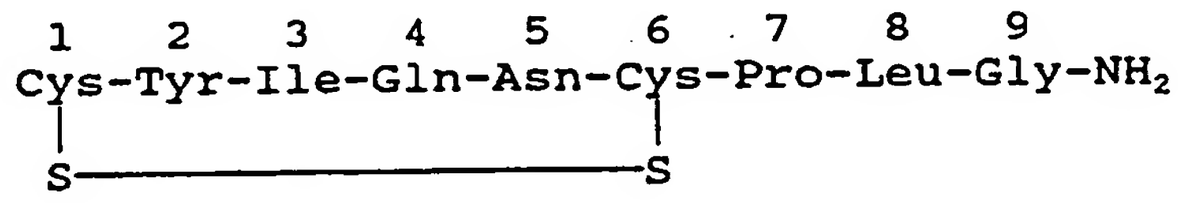
FIELD OF INVENTION

The present invention relates to a novel compound which is highly active as an oxytocin antagonist and which exhibits slight antagonism for vasopressin.

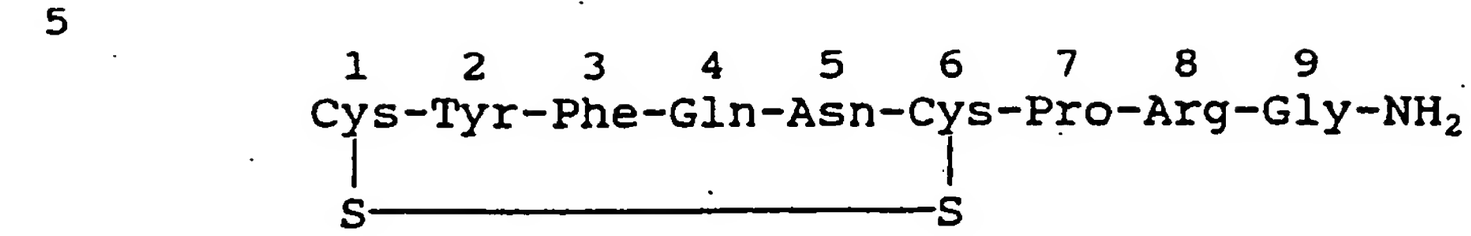
BACKGROUND OF INVENTION

Preterm labor is the major cause of prenatal morbidity and mortality in the United States. Current methods of inhibiting preterm labor are not always successful and are often associated with significant side effects. Since the uterus is a target organ for oxytocin, and assuming that oxytocin is an important contributing factor to preterm labor, the development of a potent oxytocin antagonist would result in successful inhibition of preterm labor with few associated side effects.

Structurally, oxytocin (OT) and antidiuretic hormone (ADH), also called vasopressin, are similar. Their comparative structures are illustrated below.



OXYTOCIN (OT)



VASOPRESSIN (ADH)

Various investigations in the literature have reported the synthesis of antagonists to ADH for the treatment of hypertension and the synthesis of antagonists to oxytocin. In 1960, Law, H.D. and V. DuVigneaud, J. Am. Chem. Soc., 82:4579, reported the first synthesis of an oxytocin antagonist (2-O-methyltyrosine-OT). In 1967, Chan, Fear and DuVigneaud, Endocrinology, 81:1267, reported the synthesis of 1-L-Penicillamine-oxytocin and 1-deamino-penicillamine-oxytocin. This was the first study to show an in vivo inhibitory effect of an oxytocin antagonist on uterine contractions and response to oxytocin in the anesthetized rat.

In 1980, Sawyer, et al., Endocrinology, 106:81, reported the synthesis of an oxytocin antagonist that combined the two important features of the antagonist of Law and DuVigneaud and of the antagonist of Chan, et al.. The new antagonist was (1-deamino-penicillamine, 2-O-methyltyrosine) oxytocin. The new antagonist had a pA_2 of 7.8 as determined by the oxytocic bioassay. The pA_2 is the negative logarithm of the molar concentration of the antagonist that reduces the response to the antagonist by 1/2. It is defined by Schild, British J. Pharmacology, 2:189 (1947).

In 1983, Manning, et al., J. Med. Chem., 26:1607-

161 reported the synthesis of a number of antagonists to ADH. One of these antagonists proved to have potential anti-oxytocic activity [β,β-pentamethylene-β-mercaptopropionic acid^1,D-Phe^2,Ile^4] arginine vasopressin with a pA_2 of 8.2, or in other words, 2.5 times more potent than the antagonist reported by Sawyer, et al. in 1980 (see page 1610, Table I, compound no. 1). This oxytocin antagonist can be called [Pmp^1,D-Phe^2,Phe^3,Ile^4,Arg^8] oxytocin. A related oxytocin antagonist, [Pmp^1,D-Trp^2,Phe^3,Ile^4,Arg^8] oxytocin was disclosed by Wilson and Flouret, Abstract for Society for the Study of Reproduction Meeting July 14-17, 1986.

In 1981, Melin, et al., Endocrinology, 88:173, developed an oxytocin antagonist for inhibiting preterm labor. They synthesized 1-deamino, ethyloxytocin which had a pA_2 of 7.2. They also showed that this compound inhibited uterine contractions in rats in vivo and in humans in vitro and in vivo (Akerland, et al., Obstet. and Gynecol., 62:309, 1983). In 1985, Akerland, et al., Obstet. and Gynecol. Scand., 64:499, reported the synthesis of 1-deamino[D-Tyr(OEt)^2,Thr^4, Orn^8] vasopressin with a pA_2 of 8.3. They have tested this compound in vitro on human uterine tissue and have shown it to inhibit uterine contractions.

United States Patent 4,597,901 discloses the class of vasopressin antagonists in which cysteine-1 is present in both oxytocin and vasopressin and substituted with β,β-cylopentamethylene-β-mercaptopropionic acid.

Other amino acids of vasopressin are substituted. The resulting class of compounds is said to be vasopressin antagonists the biological activity being

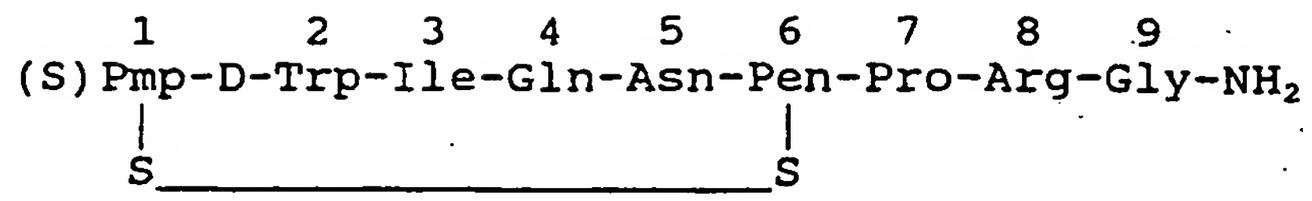
manifested as water diuresis.

SUMMARY OF INVENTION

The present invention comprises an oxytocin antagonist which is an analog of oxytocin. In the compound of this invention, cysteine-1 of oxytocin is substituted with β,β-(3-thiapentamethylene)-β-mercaptopropionic acid. In addition, L-tyrosine-2 is substituted with D-tryptophan, and penicillamine is substituted for l-cysteine in the 6 position and L-arginine is substituted in the 8 position for L-leucine. The resulting compound [(S)Pmp^1,D-Trp^2,Pen^6,Arg^8] oxytocin is believed to be novel and has been found to have remarkable properties. It is highly active as an oxytocin antagonist. At the same time, and although it is structurally similar to vasopressin and vasopressin antagonists described in the literature, the new compound exhibits minimal ADH antagonism. When these two antagonisms are expressed as a ratio, the compound of this invention has a very high anti-oxytocin/anti-ADH activity ratio. This combination of properties is highly advantageous for therapeutic use. Effective anti-oxytocin action can be obtained with minimal anti-ADH side effects. The compound of this invention is therefore adapted for inhibiting contraction of the uterine muscle in response to bodily oxytocin, and can be used to suppress preterm labor.

DESCRIPTION OF THE INVENTION

The oxytocin antagonist of this invention is represented by the formula:



wherein Pmp is β,β-(3-thiapentamethylene)-β-mercaptopropionic acid, D-Trp is the D form of tryptophan, and Ile, Gln, Asn, Pen (Pen = penicillamine), Pro, Arg, are the L forms of isoleucine, glutamine, asparagine, proline and arginine, respectively.

The remarkable properties of the novel compound of this invention are shown by bioassays, which will now be described.

Oxytocin Bioassay

The protocol used for the oxytocin bioassay procedure is derived from procedures described in a paper by Sawyer, et al., Endocrinology, 106:81 (1980), which in turn was based on reports of Munsick, Brit. J. Pharmacol., 3:328 (1960), and Holton, Brit. J. Pharmacol., 3:328 (1948). The assay calculations for the pA_2 estimates are described by Schild, British J.Pharmacology, 2:189 (1947). The major difference in the present procedure from those reported by others in the field is that the area under the contraction is integrated where most other techniques calculate the amplitude. Integration provides much more consistent and reliable results although the pA_2 estimates are approximately an order of magnitude lower than those reported using amplitude of the contraction as the endpoint.

Method:

1. Animals - a 1.5 cm piece of uterus from a virgin rat (Holtzman) in natural estrus is used for

the assay.

2. Buffer/Assay Bath - The buffer used is Munsicks. This buffer contains 0.5 mM Mg^{++} which reduces the pA_2 estimates, but the results are reported to correlate better with in vivo data (Sawyer, et al., 1980). The buffer is gassed continuously with 95% oxygen; 5% carbon dioxide giving a pH of 7.4. The temperature of the assay bath is 37 °C. A 10 ml assay bath is used that contains a water jacket for maintaining the temperature and inlet and outlet spikets for adding and removing buffer.

3. Polygraph/transducer - The piece of uterine tissue used for the assay is anchored at one end and connected to a Statham Strain Gauge Force Transducer at the other end which in turn is attached to a Grass Polygraph Model 79 for monitoring the contractions.

4. Assay Protocol. (a) The tissue is equilibrated in the assay bath for one hour with washing with new buffer every fifteen minutes. One gram of tension is kept on the tissue at all times.

(b) The tissue is stimulated initially with oxytocin at 10 nM to "acclimate" the tissue and with 4 mM KCl to determine the maximum contractile response.

(c) A cumulative dose response curve is then done with oxytocin and a concentration of oxytocin equivalent to approximately 80% of the maximum is used for estimating the pA_2 of the antagonist.

(d) The tissue is exposed to oxytocin (Calbiochemical, San Diego, California) for one minute and washed out. There is a three minute interval before addition of the next dose of the agonist or antagonist. When the antagonist is tested, it is given five minutes before the agonist. The agonist is given for one minute. All responses are integrated

using a 7P10 Grass Integrator. This is the major difference between the present protocol and others in the literature which usually measure amplitude of the contractions as the response. A single concentration of oxytocin, equal to 80% of the maximum response, is used to test the antagonist. Three different concentrations of antagonists are used, two that will reduce the response to the agonist by less than 50% and one that will reduce the response greater than 50% (ideally this relation would be 25%, 50% and 75%). This is repeated three times for each dose of antagonist for a three point assay.

(e) Calculations for pA_2: The dose-response (DR) ratios are calculated for antagonist and a Schild's Plot is performed by plotting the Log (DR-1) vs. Log of antagonist concentration. The line plotted is calculated by least squares regression analysis. The pA_2 is the concentration of antagonist at the point where the regression line crosses the 0 point of the Log (DR-1) ordinate. The pA_2 is the negative Log of the concentration of antagonist that will reduce the response to the agonist by one-half.

As an analog of oxytocin, the novel compound of this invention may be designated as [(S)Pmp^1,D-Trp^2,Pen^6,Arg^8] oxytocin. When this compound was tested by the above-described assay for competitive antagonism with oxytocin, in an average of ten assays, the pA_2 value was found to be greater than 8.86.

ADH-Bioassay

The above compound was also tested for antagonism to vasopressin. Anti-ADH activity can be determined by measuring the alteration in urine output due to ADH in the presence and absence of the antagonist. A

suitable ADH-assay is described in Sawyer, et al., Endocrinology, 63:694 (1958). When tested by this method, it was found that the compound [(S)Pmp[1], D-Trp[2], Pen[6], Arg[8]] oxytocin exhibited very low activity as a vasopressin antagonist. The ratio of oxytocin antagonism to ADH antagonism was very high, viz. over 1,866, as compared with 200 for the compositions disclosed in the parent applications.

By virtue of its oxytocin antagonist activity with minimal vasopressin antagonism, the compound of this invention will be useful in treating symptoms requiring an oxytocin antagonist in humans and animals. It can be used to inhibit uterine contractions and milk letdown as well as to inhibit preterm labor. Although the structure of the compound resembles both oxytocin and vasopressin, it exhibits not only increased anti-oxytocin activity but also greatly decreased anti-ADH activity. This compound might also be useful for inhibiting dysmenorrhea or serving as an antidote for over stimulation of uterine contraction during labor induction with oxytocin or for treating hypertension.

The compound of this invention can be administered to women by various known routes of administration. For hospital use, intravenous infusion will usually be the administration route of choice. However, the compound may also be administered intraperitoneal, subcutaneously, or intramuscularly. Oral administration may also be feasible. If required, tablets or capsules for oral use may be provided with an enteric coating protecting the compound from destruction in the stomach while permitting its release in the intestinal tract. The sublingual administration by providing suitable doses

of this compound in tablet triturates placed under the tongue may also be practical. This is the way the hormone oxytocin is given to induce milk let-down from the breasts of the lactating mother.

An effective but nontoxic quantity of the compound is employed in this treatment. The dosage regimen for preventing or treating symptoms by the compound of this invention is selected in accordance with a variety of factors including the type, age, weight, sex and medical condition of the woman, the severity of the symptoms and the route of administration of the compound. An ordinary medical practitioner can determine and prescribe the effective amount based on the route of administration of the oxytocin antagonist to prevent or arrest the progress of the condition to be inhibited. For example, an effective dose range may range from 0.01 to 100 milligrams per kilogram of body weight per day using administration by the intravenous route, such as in sterile normal saline.

The compound of this invention may be prepared by a novel method. The substitution of tryptophan in peptides may have been avoided in the past because Trp-peptides are acid sensitive. Bodanszky, et al., J. Med. Chem., 23:1258-1261 (1980) and Sawyer, et al., Endocrinology, 106:81 (1980) made [Trp-^{8}] oxytocin by more difficult indirect methods, in order to avoid acid treatment of the Trp-peptide. The methods disclosed in U.S. Serial Nos. 07/289,780 and 07/433,644 are hereby incorporated by reference.

Example I-Synthesis of [(S)Pmp^{1},D-Trp^{2},Pen^{6},Arg^{8}] oxytocin

Synthesis of β-mercaptopropionic acid

derivatives. Tetrahydrothiopyran-4-one, is reacted with triethylphosphonoacetate by the method of Wadsworth and Emmons (Wadsworth, W.S., Jr. Emmons, W.D. (1973) in Organic Synthesis (Baumgarten, H.ed.) Col. Vol. V, pp. 547-549, John Wiley & Sons, NY), yielding ethyl 4-tetrahydrothiopyranylidene (TEP)acetate. Michael addition of 4-methylbenzyl mercaptan by the method of Yim and Huffman (Yim, N.C.F. & Huffman, W.F. (1983) Int. J. Pept. Prot. Res. 21, 568-570) and saponification yields tetrahydrothiopyranyl-4-(4-methyl-benzylthio)-4-acetic acid, or (S)PmP(S-Meb). See Fig. 1.

The abbreviations used comply with recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature (J. Bio. Chem. 264, 688-673, (1989)). Where not indicated, amino acids are of the L-configuration. Other abbreviations used are: OT, oxytocin; Pmp, β,β-pentamethylene-β-mercaptopropionic acid; (S)Pmp, β,β-(3-thiapentamethylene)-β-mercaptopropionic acid; Boc, tert-butyloxycarbonyl; Meb, 4-methylbenzyl; Tos, p-toluenesulfonyl; ONp, 4-nitrophenyl ester; DCM, dichloromethane; TFA, trifuoroacetic acid; EtOH, ethanol; DIEA, diisopropylethylamine; DMF, dimethylformamide; DCC, dicyclohexylcarbodiimide; HOBt, 1-hydroxy Benzotriazole; MeOH, methanol; CHL, chloroform; Ac_2O, acetic anhydride; TEA, triethylamine; MeCN, acetonitrile; BuOH, n-butanol; AcOH, acetic acid; Pyr, pyridine; Et_2O, ethyl ether; HPLC, high performance liquid chromatography; TLC, thin layer chromatography; PITC, phenylisothiocyanate; PTC, phenylthiocarbamyl; UV, ultraviolet; OR, optical rotation.

Peptide synthesis. All protected peptides

precursors of the antagonists were synthesized manually by the solid phase (SP) method (Merrifield, R.B. (1963) J. Am. Chem. Soc. 85, 2149-54). The Boc-amino acids (Stewart, J.M. & Young, J.D. (1984) in Solid Phase Peptide Synthesis pp. 1-176, Pierce Chemical Co., Rockford, IL) strategy of synthesis was followed. All position 1 analogs of Pmp had the thiol group protected with the 4-methylbenzyl group. Completion of coupling was monitored by means of the ninhydrin test (Kaiser, E., Colescot, R.L., Bossinger, C.D. & Cook, P.I. (1970) Anal. Biochem. 34, 595-598). Protected peptides were removed from the resins by ammonolysis (Manning, M., (1968) J. Am. Chem. Soc. 90, 1348-1349). Protected peptides were freed from blocking groups on side chain functionalities by reduction with Na/liquid ammonia (du Vigneaud, V., Ressler, C., Swan, J.M., Roberts, C.W., Katsoyannis, P.G. & Gordon, S. (1953) J. Am. Chem. Soc. 75, 4879-4880) or liq HF-anisole (Sakakibara, S. & Shimonishi, Y. (1965) Bull. Chem. Soc. Jpn. 38, 1412-1413) and the disulfhydryl peptides were cyclized in very dilute solution (Manning, M., Lammek, B. & Kolodziejczyk, A.M. (1981) J. Med. Chem. 24, 701-706) to the cyclic disulfide by oxidation with potassium ferricyanide (Hope, D.B., Murti, V.V.S. & du Vigneaud, V. (1962) J. Biol. Chem. 237, 1563-1566). The free peptides were freed from small by-products and salts by gel filtration (Porath, J. & Flodin, P. (1959) Nature (London) 183, 1657-1659) on Sephadex G-15 (Manning, M., Wuu, T.C. & Baxter, J.W.M. (1968) J. Chromatogr. 38, 396-398) and by preparative high performance liquid chromatography (HPLC) (Flouret, G., Brieher, W., Mahan, K., and Wilson, L., Jr. (1991) J. Med. Chem. 34, 642-646). Peptide purity was monitored by TLC, HPLC, and amino acid analysis (Bidlingmeier,

B.A., Cohen, S.A. & Tarvin, T.L. (1984) J. Chromatogr. 336, 93-104).

The peptide sequence of each analog was assembled manually by the SP method using a mechanical shaker and a special vessel. Where suitable some peptides were deprotected with liquid HF, using an all-Teflon apparatus (Protein Research Foundation, Osaka, Japan). Boc-amino acids were supplied by Bachem, and synthetic or ionic resins were supplied by BioRad. all other reagents were supplied by Aldrich Chemical Co., Pierce, or Chemical Dynamics. The purity of peptides was checked by analytical HPLC with a Millipore apparatus previously described (Flouret, G., Brieher, W., Mahan, K., and Wilson, L., Jr. (1991) J. Med. Chem. 34, 642-646) and an analytical μBondapak C_{18} column (30 x 0.39 cm). For preparative HPLC we used a Gilson auto-preparative HPLC System 71 as previously described (Flouret, G., Brieher, W., Mahan, K., and Wilson, L., Jr. (1991) J. Med. Chem. 34, 642-646) and a preparative column module 21.4 x 25 cm, with a guard module, 5 cm, both modules packed with Dynamax-60A, 8 μm, C_{18} (Rainin). The solvents used for chromatography or synthesis were HPLC grade (Fisher Scientific). The solvent systems used both for analytical or preparative HPLC were: (a) 0.05% TFA; (b) 60% MeCN-40% solvent A. The purity of peptides was also monitored by thin-layer chromatography (TLC) on silica gel G pre-coated Uniplates (0.25 mm, Analtech). The solvent systems used (ratios given by volume) were: (A) n-BuOH-AcOH-H_2O (4:1:1); (B) n-BuOH-AcOH:H_2O (4:1:5, upper phase); (C) n-BuOH-AcOH:H_2O (5:1:1); (D) n-BuOH-AcOH:H_2O:Pyr (5:1:1:1). Peptides were visualized with Ehrlich reagent or chlorine-tolidine (Stewart, J.M. & Young, J.D. (1984)

in Solid Phase Peptide Synthesis pp. 1-176; Pierce Chemical Co., Rockford, IL). For amino acid analysis analogs were hydrolyzed with 6N HCl for 24 hr. at 110°C and the resulting amino acid components were derivatized with Phenylsiothiocyanate and analyzed by the Waters Associates Picotag method (using a Waters Picotag set up as previously described (Flouret, G., Brieher, W., Mahan, K., and Wilson, L., Jr. (1991) J. Med. Chem 34, 642-646). The optical rotations of peptides were measured with a Rudolph Polarimeter (precision ± 0.01°).

Solid-Phase Synthesis of Protected Peptides. Boc-amino acids were used for the synthesis, and for protection of side chain functionalities, Boc-Arg(Tos), Boc-Pen(Meb), and (S)Pmp(Meb). We used Boc-Gly-Resin (0.7 mmol of Boc-Gly/g) which was prepared on a 200-400 mesh cloromethylated resin (BioRad), 1% cross-linked with divinylbenzene, by esterification with the cesium salt of respective Boc-amino acid (Gisin, B.F. (1973) Helv. Chim. Acta 65, 1476-1482). The Boc-Gly-Resin (0.5-0.7 mmol/g) was taken manually through the required number of coupling cycles by the SP method of synthesis as previously modified (Flouret, G., Brieher, W., Mahan, K., and Wilson, L., Jr. (1991) J. Med. Chem. 34, 642-646). In each cycle the Boc group was removed with 30% trifluoroacetic acid in DCM and, after neutralization of the resin with 10% DIEA in DCM, coupling was performed with a three-fold excess of Boc-amino acid and DCC. Six molar excess of Boc-Asn-ONp or Boc-Gln-ONp in DMF was used at the appropriate steps, and the excess reagent was recovered by precipitation with water. Completion of the coupling step was monitored by means of the ninhydrin test which usually gave a negative response. If the test was positive, the coupling step was

repeated, but if only faintly positive, the peptide was capped by acetylation with Ac_2O:DIEA:DCM (1:1:8). Unprotected Boc-D-Trp was introduced at position 2. The Boc-group was then removed with 30% TFA in DCM containing 1% mercaptoethanol and 10% anisole and (S)Pmp(S-Meb) was incorporated in 3 mole excess in DMF solution by activation with DCC and HOBt. The final assembled peptide was removed from the resin by ammonolysis with MeOH (25 ml) saturated with ammonia. After 3 days, the resin was removed by filtration, and extracted three times with hot DMF. The methanolic filtrate and the DMF extracts were pooled and evaporated to dryness. The residue was dissolved in DMF (2-3 ml) and the protected peptide amide was precipitated from the pooled DMF extracts by treatment with water or EtOH:Et_2O, yielding 400-600 mg of protected peptide. TLC analysis of protected peptides obtained after ammonolysis usually showed one major component with minor impurities, hence, they were used directly for deprotection and preparation of the free analogs.

Ethyl-4-tetrahydrothiopyranylidene acetate: This ester was prepared as described for the preparation of ethyl-4-tetrahydropyranylidene acetate (Wadsworth, W.S., Jr. Emmons, W.D. (1973) in Organic Synthesis (Baumgarten, H. ed.) Coll. Vol. V, pp. 547-549, John Wiley & Sons, New York) and vacuum distillation, oil (73% yield).

Tetradrothiopyranyl-4-(4-methyl-benzylthio)-4-acetic acid, or (S)Pmp(4-S-Meb). This protected acid was prepared from the preceding ester, by the method of Yim and Huffman as described for Pmp (Int. J. Pept. Prot. Res. 21, 568-570, 1983), mp. 113-115° (50-70% yield).

[(S)Pmp^1,D·Trp^2,Pen^6,Arg^8]OT, or Antag III.

(S)Pmp(S-Meb)-D-Trp-Ile-Gln-Asn-Pen(Meb)-Pro-Arg(Tos)-Gly-NH_2 (600 mg), assembled by the SP method (starting with 0.5 mmole of amino acid-resin) as described above, was dissolved in liquid ammonia (200 ml) freshly distilled from sodium and treated under anhydrous conditions with a sodium stick until a pale blue color lasted for about 15-30 sec. After evaporation of ammonia in a vacuum, the solid residue was dissolved in 20 ml of 50% AcOH. This dissolved peptide was added to deaerated water (2 L) (this large volume can be sharply reduced by a modified procedure) the pH was adjusted to 7.0 by the addition of concentrated ammonium hydroxide and cyclization to the peptide disulfide was brought about by titration of the disulfhydryl peptide with 0.01N potassium ferricyanide until a permanent yellow color resulted and then adding 20% excess of potassium ferricyanide solution. After 20 min, the ferrocyanide and ferricyanide salts were removed by stirring for 10 min with AG1 X-2 (Cl^-) ion exchange resin (15 g.) and then by passing the suspension through a column containing additional ion exchange resin (15 g.), using additional 0.2N AcOH (100 ml) for washings. The combined filtrate and washings were lyophilized. Analysis of the solid obtained containing the peptide was accomplished on an analytical μBondapak C_{18} column (30 x 0.39 cm), monitoring at 220 nm, and eluting isocratically with 55% solvent B (solvent A, 0.05% TFA; solvent B 60% MeCN-40% of 0.05% TFA), at a rate of 1.8 ml/min. Under these conditions there was good resolution of impurities. The residue was dissolved in the smallest possible volume of 50% acetic acid and was applied to a Sephadex G-15 column (115 x 2.7 cm)

and eluted with the same solvent at a rate of about 50-60 ml/hr (6). The eluate was monitored in a UV spectrophotometer at 254 nm. The fractions corresponding to the major peak were monitored by analytical HPLC, with an analytical μBondapak C_{18} column (30 x 0.39 cm), eluting with 57% solvent B, and detecting peptides at 220 nm. The pure fractions by HPLC criteria were pooled and lyophilized. The residue was dissolved in 0.2 N AcOH (20 ml) and was applied to a preparative Dynamax-60A, 8 μm, C_{18} (Rainin) column, 21.4 x 25 cm, with a 5 cm guard module. A gradient was run from 0 to 45% B over 45 minutes, eluting at a rate of 5 ml/min, monitoring the eluent at 280 nm. Center portions of the main component eluted after approximately 3.5 hr. The purer fractions determined by analytical HPLC, were pooled and lyophilized, yielding Antag III (240 mg, 42% from initial resin). Analogue purity was established by thin layer chromatography (TLC) in four separate solvent systems, by analytical HPLC, and by amino acid analysis. The analogue gave the expected amino acid analysis ratios ± 10%. D-Tryptophan in peptides, was estimated by UV spectrophotometry at 280 nm (13). The lower value found for tryptophan, 0.96, suggests that the peptide lyophilisate may have TFA, and/or H_2O.

TABLE 1

[alpha]$D^{27°}$-39°(1N, AcOH)

TLC:	(A)	n-BuOH-AcOH-H_2O (4:1:1)	Rf 0.27
	(B)	n-BuOH-AcOH:H_2O (4:1:5, upper phase)	0.42
	(C)	n-BuOH-AcOH:H_2O (5:1:1)	0.19
	(D)	n-BuOH-AcOH:H_2O:Pyr (5:1:1:1)	0.56

EXAMPLE II-Comparative testing of Compounds

For comparison with [(S)Pmp^1,D-Trp^2,Pen^6,Arg^8] oxytocin (antagonist D in Table 2), three related compounds were synthesized. One of these was the compound described by Manning, et al., J. Med. Chem., 26:1607-1613 (1983). This compound can be called [Pmp^1,D-Phe^2,Phe^3,Ile^4, Arg^8] oxytocin. This compound is called antagonist A in Table 2. The other compound was [Pmp^1,D-Trp^2, Phe^3,Ile^4,Arg^8] oxytocin was disclosed in U.S. no. 07/239,780 and is referred to as antagonist B in table 2. The third comparative compound [Pmp^1, D-Trp^2, Arg^8] oxytocin was disclosed in U.S. serial No. 07/433,664 and is referred to as antagonist C in Table 2. The four compounds were comparatively studied in bioassays.

Oxytocic Bioassay.

The protocol used for the oxytocin bioassay procedure is derived from procedures described in a paper by Sawyer, et al., Endocrinology, 106:81 (1980), which in turn was based on reports of Munsick, Brit. J. Pharmacol., 3:328 (1960), and Holton, Brit. J. Pharmacol., 3:328 (1948). The assay calculations for the pA_2 estimates are described by Schild, Brit. J. Pharmacol. (1947). The major difference in procedure from those previously reported was the integration of the area under the contraction instead of merely calculating the amplitude. Integration provides more consistent and reliable results, although the pA_2 estimates are about an order of magnitude lower than those reported using amplitude of the contraction as the endpoint.

Method: Animals. A 1.5 cm piece of uterus from a virgin rat (Holtzman) in natural estrus is used for the assay.

Buffer/Assay Bath. The buffer used is Munsick's. This buffer contains 0.5 mM Mg^{++} which reduces the pA_2 estimates, but the results are reported to correlate better with the in vivo data (Sawyer, et al., 1980). The buffer is gassed continuously with 95% oxygen:5% carbon dioxide giving a pH of 7.4. The temperature of the assay bath is 37°C. A 10 ml assay bath is used that contains a water jacket for maintaining the temperature and inlet and outlet spikets for adding and removing buffer.

Polygraph/Transducer. The piece of uterine tissue used for the assay is connected to a Statham Strain Gauge Force Transducer which in turn is attached to a Grass Polygraph Model 79 for monitoring the contractions.

Assay Protocol. (a) The tissue is equilibrated in the assay bath for one hour with washing with new buffer every 15 minutes. One gram of tension is kept on the tissue at all times.

(b) The tissue is stimulated initially with oxytocin at 10 nM to "acclimate" the tissue and with 4 mM KCl to determine the maximum contractile response.

(c) A cumulative dose response curve is then determined with oxytocin and a concentration of oxytocin equivalent to approximately 80% of the maximum used for estimating the pA_2 of the antagonist.

(d) The tissue is exposed to oxytocin (Calbiochemical) for one minute and washed out. There is a three minute interval before addition of the next dose of the agonist or antagonist. When the antagonist is tested, it is given five minutes before

the agonist. The agonist is given for one minute. All responses are integrated using a 7P10 Grass integrator. This is the major difference between our protocol and others in the literature who usually measure amplitude of the contractions as the response. A single concentration of oxytocin, equal to 80% of the maximum response, is used to test the antagonist. Three different concentrations of antagonists are used, two that will reduce the response greater than 50% (ideally this relation would be 25%, 50% and 75%). This is repeated three times for each dose of antagonist for a three point assay.

The anti-ADH activity is measured by the alteration in urine antagonist by ADH in the presence and absence of the antagonist to determine the specificity of the antagonist. The anti-ADH assay is described in Sawyer, et al., Endocrinology, 63:694 (1958).

Additional studies were performed to determine if the results of the rat bioassays reflected the binding affinity to the uterine OT receptors in the rat and human. The relative binding affinities of 5 different oxytocin antagonists were compared.

Oxytocin Receptor Assays.

Method. Rats. Uterine tissue was removed on day 21 of pregnancy (delivery = Days $21_{1/2}$ to $22_{1/2}$) from Holtzman rats. The tissue was emptied of its contents, rinsed in ice cold buffer, cut into small pieces and frozen at -70°C until homogenization.

Humans. Human myometrial tissue was collected from patients at the time of cesarian section after informed consent. The tissue was rinsed in cold buffer, cut into small pieces and frozen at -70°C until homogenization.

Isolation of oxytocin receptors. Oxytocin receptors (OTrs) reside on the cell membrane and are present at high concentrations at the end of pregnancy in uterine tissue. Frozen tissue is homogenized in Tris buffer, the homogenate filtered, and the filtrate centrifuged at 1000 g for 15 minutes at 4°C. The supernatant is centrifuged at 40,000 g for 30 minutes and the pellet containing the cell membranes resuspended in 10% sucrose. Density gradient ultracentrifugation is then performed by placing the 10 % sucrose suspension on top of 35% sucrose and centrifuging for 30 minutes in a swing bucket rotor at 105,000 g. The membranes at the interface of the 10%/35% sucrose are removed and resuspended in Tris buffer containing EDTA for 30 minutes. This procedure removes divalent cation and results in dissociation of any endogenously bound OT to the receptor. This mixture is then centrifuged for 15 minutes at 100,000 g and the pellet containing the membrane OTrs resuspended in Tris, PMSF, Mg^{++}, buffer by sonication.

OT receptor assay. The binding assay consist of 0.1 ml of 20,000 cmp of tritium labeled OT (New England Nuclear, 37.1 Ci/nmol), 0.1 ml of OT antagonist added at increasing concentration, 0.25 ml of buffer and 0.05 ml of membrane (70-150 ug protein). A nonspecific tube has 100X of cold OTA added to it. The incubation is for 30 minutes at 30 °C. The membrane is pelleted by centrifugation in ultraclear mini tubes (5 x 41 mm) for 30 minutes at 105,000 g. The resulting pellet containing the bound ^{3}H-OT is dissolved in 0.1 N NaOH at 45 °C for 30 minutes and this mixture is then placed in liquid scintillation counting fluid and counted for dpms in a scintillation counter.

The data is analyzed by nonlinear curve fitting methods using McPherson's EBDA (J Pharmacol Methods 14:213-228, 1985) and Munson and Rodbard's LIGAND (Anal Biochem 107:220-239, 1980) program for saturation and competition analysis for determining Kds and Kis.

Results of the comparative bioassay and receptor studies are shown in Tables 2-4.

TABLE 2

	Oxytocic Bioassay		ADH Bioassay		
OTA*	pA_2	Relative Anti-Oxytocic Activity	pA_2	Relative Anti-ADH Activity	Ratio Anti-OT AntiADH
A	7.35	0.7	7.66	1.000	0.70
B	7.51	1.0	7.40	0.550	1.82
C	7.77	1.7	5.51	0.007	242.86
D	8.86	22.4	< 5.75	0.012	>1866.7

*Compound A is [Pmp^1,D-Phe^2,Phe^3,Ile^4,Arg^8] oxytocin, as described by Manning, et al., J. Med. Chem., 26:1607-1613 (1983).
Compound B is [Pmp^1,D-Trp^2,Phe^3,Ile^4,Arg^8] oxytocin = ANTAG I
Compound C is [Pmp^1,D-Trp^2,Arg^8] oxytocin = ANTAG II.
Compound D is [(S)Pmp^1,D-Trp^2,Pen^6,Arg^8] oxytocin = ANTAG III.

In Table 2, Compound D, comprising the novel compound of this invention, demonstrated a higher anti-oxytocic activity than of the other three compounds. Further, it had a much lower anti-ADH

activity. The ratio of anti-OT/anti-ADH for Compound D is greater than 1,866.70, while this ratio for Compound A was 0.7, Compound B 1.8 and Compound C 242.9. These data therefore indicate that Compound D can be expected to produce less anti-ADH side effects when administered at an effective oxytocin antagonist dose than either Compounds A, B or C.

Table 3 illustrates the comparison of the binding affinities (Kas) estimated from the rat uterine receptor assay (Kas) versus the bioassay. Correlation of the Log_{10} Ka with Log_{10} ED_{50} was highly significant ($r = 0.92$; $p<0.01$). Comparison of the relative activity of ANTAG III (compound D) to ANTAG I (compound B) by the rat uterine receptor assay and bioassay is shown in Table 5. By both assays ANTAG III is approximately 20X more potent than ANTAG I.

Table 4 shows the binding affinity of the different OTAs to the human uterine OTr compared to the rat bioassay. Correlation of the Log_{10} Ka to the Log_{10} ED_{50} was highly significant ($r = 0.95$; $p<0.01$). Comparison of the relative binding activity of ANTAG III versus ANTAG I to the human OTr (hOTr) is shown in Table 5. By this estimate ANTAG III (compound D) is about 80X more potent than ANTAG I. Therefore, it appears that the rat assays might be under estimating the relative potency of ANTAG III in the human. This possibility is further supported by the <u>in vivo</u> studies performed in the pregnant baboon described below.

<u>Example III testing of Compounds in Pregnant Baboons</u>

The purpose of this study was to ascertain the relative <u>in vivo</u> activity of four oxytocin antagonist using the tethered pregnant baboon model and compare these results to previous activity estimates using rat

assays and human OTr assays. The baboon is an excellent animal model because of its physiologic and anatomic similarity to humans (see articles by our laboratory: Am J Obstet Gynecol. 163:1815-1882, 1990; Am J Obstet Gynecol 165:456-560, 1991; Am J Obstet Gynecol 165:1487-1498, 1991; articles by other laboratories: Endocrine Reviews 11:124-150, 1990; 11:151-176, 1990). Pregnant tethered baboons were studied between 130 to 145 days of pregnancy Delivery = day 184). The oxytocin antagonists were administered as a single bolus injection of 1 mg intra-arterially followed 1 minute later by the infusion of oxytocin. Oxytocin was infused continuously beginning at 10 mU and doubling the dose every 20 minutes up to 400 mU/minute or until the contractile force (CF=(freq x mean amplitude)/10 minutes] response was significant (ie CF>50). If there was no significant response the oxytocin challenge test was repeated 24 hours later. The antagonists-response interval (ARI) was determined by multiplying the time to the first significant response in minutes by the contraction to pulse ratio (CF/OT concentration). Results: The results are shown in Table 5. The ARI was highly correlated with the rat and human OTr estimates of binding affinity (Ka) (r-0.98; p<.01) with 4/5 oxytocin antagonists. One oxytocin antagonist (antagonist F in Table 5) showed no inhibitory activity at the dose tested although it had moderately good binding affinity in the rat and human OTr assays and rat bioassay. This oxytocin antagonist was not produced in our laboratory and is not an oxytocin analog. One mg of the best oxytocin antagonist tested (Compound D, the novel compound of this invention) blocked the response to oxytocin antagonist for greater than 24 hours. Comparison of

the relative activities of the different OTAs to compound B (ANTAG I) suggests that compound D, [(S)PMP^1,D-Trp^2,Pen^6,Arg^8] oxytocin (ie ANTAG III) is about 130 times more potent than compound B. In summary, the relative activity ratios (see Table 5) of ANTAG III (D) to ANTAG I (B) by rat bioassay, rat receptor assay, human receptor assay and in vivo baboon bioassay were 22, 20, 82 and 133, respectively. These data indicate that the rat assays might be underestimating the potency of ANTAG III in primates.

TABLE 3. COMPARISON OF RELATIVE UTERINE RECEPTOR BINDING (Ka) AND BIOASSAY INHIBITORY ACTIVITY (ED50) FOR OXYTOCIN ANTAGONISTS

	RECEPTOR ASSAY	OXYTOCIC BIOASSAY
Oxytocin Antagonist	Ka($10^{+8}M^{-1}$)	ED50 (nM)
A	0.39	44.76
B	1.16	30.90
C	2.03	16.98
C2	11.50	1.91
D	24.40	1.38
E	0.51	102.33
F	5.89	19.93

RECEPTOR ASSAY - PREGNANT RAT UTERUS (P-21)
BIOASSAY - ESTROUS RAT UTERUS

A - DESCRIBED BY MANNING ET AL. J. MED. CHEM. 26:1607, 1983
B - [Pmp^1, D-Trp^2, Phe^3, ILe^4, Arg^8] OXYTOCIN = ANTAG I
C - [Pmp^1, D-Trp^2, Arg^8] oxytocin = ANTAG II
C2 - [Pmp^1, D-Trp^2, Pen^6, Arg^8] oxytocin = ANTAG II-2
D - [(S)PMP^1, D-Trp^2, Pen^6, Arg^8] oxytocin = ANTAG III
E - [Mpa^1,D-Tyr(Et)^2,Thr^4, Orn^8] oxytocin (ie.ATOSIBAN)
F - L-366,948 FROM MERCK PHARMACEUTICAL

TABLE 4. COMPARISON OF UTERINE RECEPTOR BINDING (Ka) IN HUMAN MYOMETRIAL TISSUE TO RAT UTERINE BIOASSAY INHIBITORY ACTIVITY (ED50) FOR OXYTOCIN ANTAGONISTS

OTA	RECEPTOR ASSAY Ka($10^{+8}M^{-1}$)	BIOASSAY ED50(nM)
B	0.51	30.9
C	1.82	17.0
D	41.7	1.38
E	0.53	102.3
F	2.49	20.0

RECEPTOR ASSAY - MYOMETRIAL TISSUE TAKEN FROM WOMEN AT TERM BY C-SECTION
BIOASSAY - ESTROUS RAT UTERUS
COMPOUNDS B-F ARE DESCRIBED IN TABLE 3

TABLE 5. COMPARISON OF THE RATIOS OF BIOLOGIC ACTIVITY (ARI) OF 5 OXYTOCIN ANTAGONISTS (OTAs) IN THE PREGNANT BABOON TO THE RATIOS OF OXYTOCIN RECEPTOR (OTr) BINDING AFFINITY AND BIOASSAY ACTIVITY IN THE RAT AND HUMAN

OTA	ARI	ARI (OTA/ANTI)	rOTr (OTA/ANTI)	hOTR (OTA/ANTI)	BIOASSAY (ANTI/OTA)
B	59	1	1.0	1.0	1.0
C	547	9	1.8	3.6	1.8
D	7856	133	20.0	81.8	22.1
E	-	-	0.4	1.0	0.3
F	0	0	5.1	4.9	1.5

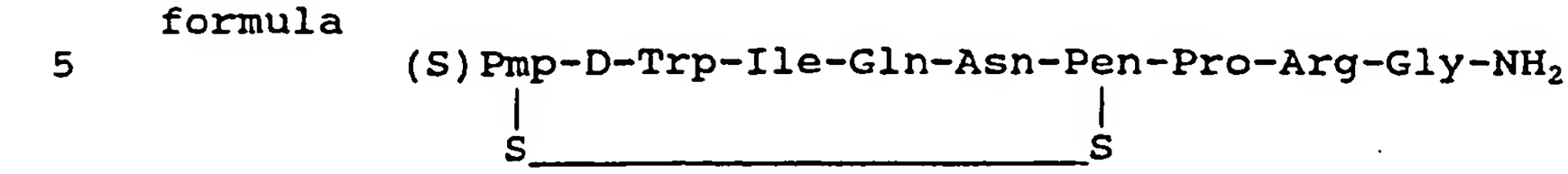
ARI - ANTAGONISTS-RESPONSE INTERVAL IN THE PREGNANT BABOON (SEE TEXT FOR EXPLANATION OF CALCULATION)
rOTr - RAT OXYTOCIN RECEPTOR
hOTr - HUMAN OXYTOCIN RECEPTOR
BIOASSAY - RAT OXYTOCIC BIOASSAY
ANTI-ANTAG I
COMPOUNDS B-F ARE DESCRIBED IN TABLE 3.

Although the invention has been described primarily in connection with special and preferred embodiments, it will be understood that it is capable of modification without departing from the scope of

the invention. The following claims are intended to cover all variations, uses, or adaptations of the invention, following in general, the principles thereof and including such departures from the present disclosure as come within known or customary practice in the field to which the invention pertains, or as are obvious to persons skilled in the field.

WE CLAIM:

1. The oxytocin antagonist represented by the formula



wherein (S)Pmp IS β,β-(3-thiapentamethylene)-β-mercaptopropionic acid, D-Trp is the D form of tryptophan, and Ile, Gln, Asn, Pen (Pen = penicillamine), Pro, and Arg are the L forms of isoleucine, glutamine, asparagine, proline and arginine, respectively.

INTERNATIONAL SEARCH REPORT

Internatior pplication No
PCT/US 94/01439

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 C07K7/16

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	JOURNAL OF MEDICINAL CHEMISTRY vol. 36, no. 6 , 19 March 1993 , WASHINGTON US pages 747 - 749 G. FLOURET ET AL. 'Systematic Substitution of an Oxytocin Antagonist with D-Amino Acids: Unexpected High Antagonistic Potency of the D-Cys6-Substituted Analogue' see compound 10 in tables I and II see page 748, right column, paragraph 3 ---	1
Y	US,A,5 070 187 (H. GAVRAS AND B. LAMMEK) 3 December 1991 see column 4, line 27 - column 5, line 42 see column 10, line 61 - column 12, line 27; claims ---	1

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 August 1994	05 -10- 1994
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	**Authorized officer** Fuhr, C

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INTERNATIONAL SEARCH REPORT

Internation pplication No
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP,A,0 216 606 (SMITHKLINE BECKMAN CORPORATION) 1 April 1987 see page 2, line 39 - line 54; claims; examples 1,3,6	1
A	WO,A,90 02756 (NORTHWESTERN UNIVERSITY) 22 March 1990 see the whole document	1

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INTERNATIONAL SEARCH REPORT

Information on patent family members

Internation pplication No
PCT/US 94/01439

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
US-A-5070187	03-12-91	NONE		
EP-A-0216606	01-04-87	US-A-	4711877	08-12-87
		AU-B-	597271	31-05-90
		AU-A-	6267586	26-03-87
		JP-A-	62067100	26-03-87
WO-A-9002756	22-03-90	AU-A-	4211089	02-04-90